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An alternative to conventional insect marking procedures: detection of a protein mark on pink bollworm by ELISA*

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Abstract

Four experiments were conducted to examine the feasibility of marking pink bollworm, *Pectinophora gossypiella* (Saunders) with rabbit immunoglobulin G (IgG) protein for mark-release-recapture studies. Pink bollworm were internally marked by feeding larvae an enriched rabbit IgG diet or externally marked by submerging pupae and spraying adults. Individuals were then assayed for the presence of rabbit IgG by sandwich enzyme-linked immunosorbent assay (ELISA) using anti-rabbit IgG. The internal marker was retained in larvae and retained in prepupae and pupae, but not in adults. A second experiment showed that rabbit IgG was retained on adults that were externally marked as pupae. A third series of tests examined the feasibility of externally marking adults with rabbit IgG. Rabbit IgG was retained on externally marked adults for six days in the field. Protein was retained on marked moths in the laboratory after they were captured on and removed from sticky traps. Finally, laboratory tests showed that large groups of externally marked moths transferred rabbit IgG to unmarked moths, but individual males do not readily transfer the protein to unmarked females in small vials.

Introduction

Studies to measure the dispersal characteristics of pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) frequently are conducted to determine dispersal patterns and behaviors (Flint et al., 1975; Van Steenwyk et al., 1978; Flint & Merkle, 1981; Bartlett, 1982; Raulston et al., 1996; Tabashnik et al., 1999). A wide variety of markers currently are available for marking pink bollworm and other lepidopterous pests prior to their release so that they can be distinguished from their native counterparts upon recapture (Hagler & Jackson, 2001). The most common marker for pink bollworm is Calco Oil Red N-1700® dye added to pink bollworm larval diet. As larvae feed on the dye-enriched diet their fat body becomes visibly marked, and they remain marked as adults (Graham & Mangum, 1971). However, dye marking is not always effective on pink bollworm (EM pers. obs.) or for certain mark-release-recapture studies where several markers (e.g., different colored markers) are needed to distinguish groups of insects released at different locations (Hayes, 1989; Hagler, 1997a; Hagler & Jackson, 2001).

A new marking procedure was developed for marking insects for mark-release-recapture studies (see Hagler references). The technique involves marking insects with the readily available protein, rabbit immunoglobulin G (IgG). In turn, marked insects are analyzed for the presence of the marker by sandwich enzyme-linked immunosorbent assay (ELISA) using anti-rabbit IgG. The protein marking technique was so effective for marking such a wide variety of insects

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that we decided to test the efficacy of protein marking on internally and externally marked pink bollworm.

Specifically, we report here on the results of a series of studies designed to determine if rabbit protein can be used as a marker: (1) in various pink bollworm life stages after larvae feed on artificial diet containing rabbit IgG, (2) on adults after externally marking pupae, (3) on adults after externally marking adults, and (4) on unmarked adults after contact with externally marked adults. The objective of this study is to determine the feasibility of marking a lepidopterous pest with protein for future mark-release-recapture studies.

Materials and methods

Test insects. Pink bollworm were obtained from the USDA-APHIS Plant Protection Center and the USDA-ARS Western Cotton Research Laboratory mass-rearing facilities located in Phoenix, Arizona. Pink bollworm reared in the USDA-APHIS facility were reared on diet described by Stewart (1984). Pink bollworm reared in the USDA-ARS facility were reared on the artificial diet described by Bartlett & Wolf (1985). All insects were maintained in an environmental chamber (27 °C, 40% r.h., and L16:D8 after marking unless otherwise noted.

ELISA procedure. A sandwich ELISA was performed on each individual as described by Hagler (1997a,b). Briefly, each well of a 96-well ELISA microplate was coated with 100-μl of anti-rabbit IgG (developed in goat) (Sigma Chemical Co., St. Louis, MO, No. R2004) diluted 1:500 in double-distilled H_2O and incubated overnight at 4 °C. The anti-rabbit IgG was discarded and 360-μl of 1% nonfat dry milk in double-distilled H_2O was added to each well for 30 min at 27 °C to block any remaining nonspecific binding sites on the plates. After the nonfat milk was removed, a 100-μl aliquot of the homogenized insect sample (see below for sample preparation) was placed in each well of the pretreated assay plate and incubated for 1 h at 27 °C. Insect samples were then discarded and each well was briefly rinsed three times with tris-buffered saline (TBS) Tween 20 (0.05%) and twice with TBS. Aliquots (50-μl) of anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, No. A-6154) diluted to 1:1,000 in 1.0% nonfat milk, were added to each well for 1 h at 27 °C. Plates were again washed as described above, and 50-μl of substrate was added using the reagents supplied in a horseradish peroxidase substrate kit (Bio-Rad, Richmond, CA, No. 172-1064). Following substrate incubation (4 h), the optical density of each sample was measured with a microplate reader set at 405 nm.

Negative control threshold calculation. Larvae, pupae, and adult pink bollworm known not to contain any rabbit IgG (negative controls) were assayed by the sandwich ELISA described above. Individually marked pink bollworm for each of the experiments described below were scored positive for the presence of the marker if its ELISA reading exceeded the mean negative control ELISA reading by three standard deviations (Hagler, 1997a, b).

Insect sample preparation. Individual pink bollworm from each test were stored at -70 °C before homogenizing in 500-μl TBS (pH 7.4), and assaying for the presence of the internal rabbit IgG marker by the ELISA described above.

Experiment 1: transfer of the rabbit IgG mark between life stages

An experiment was conducted to determine if rabbit IgG could be detected in pink bollworm larvae and their subsequent life stages after feeding on larval diet enriched with rabbit IgG. Technical grade rabbit IgG (Sigma Chemical Co., St. Louis, MO, No. I-8140) was added to pink bollworm artificial diet just prior to the solidification of the diet (Adkisson et al., 1960). The final concentration of the rabbit IgG-enriched diet was 1.13 mg rabbit IgG per ml of diet. Fourth instar larvae were removed from regular diet and placed on the rabbit IgG enriched diet. The larvae fed on the diet until they reached their prepupal stage ($\approx$ four days). At this point, the larvae ceased feeding, chewed out of their rearing containers, and fell onto clean sorting trays (Bartlett & Wolf, 1985). The pink bollworm life stages examined for the presence of rabbit IgG included larvae that fed on the rabbit IgG-enriched diet for two or four days, 1-day-old prepupae, 2-, 4-, and 7-day-old pupae, and 1-day-old adults. The mean ($\pm$ s.d.) ELISA readings and the percentage of pink bollworm scoring positive for rabbit IgG were recorded for each group.

Experiment 2: retention of protein from externally marked pupae to adults

An experiment was conducted to determine if rabbit IgG transferred from externally marked pupae to

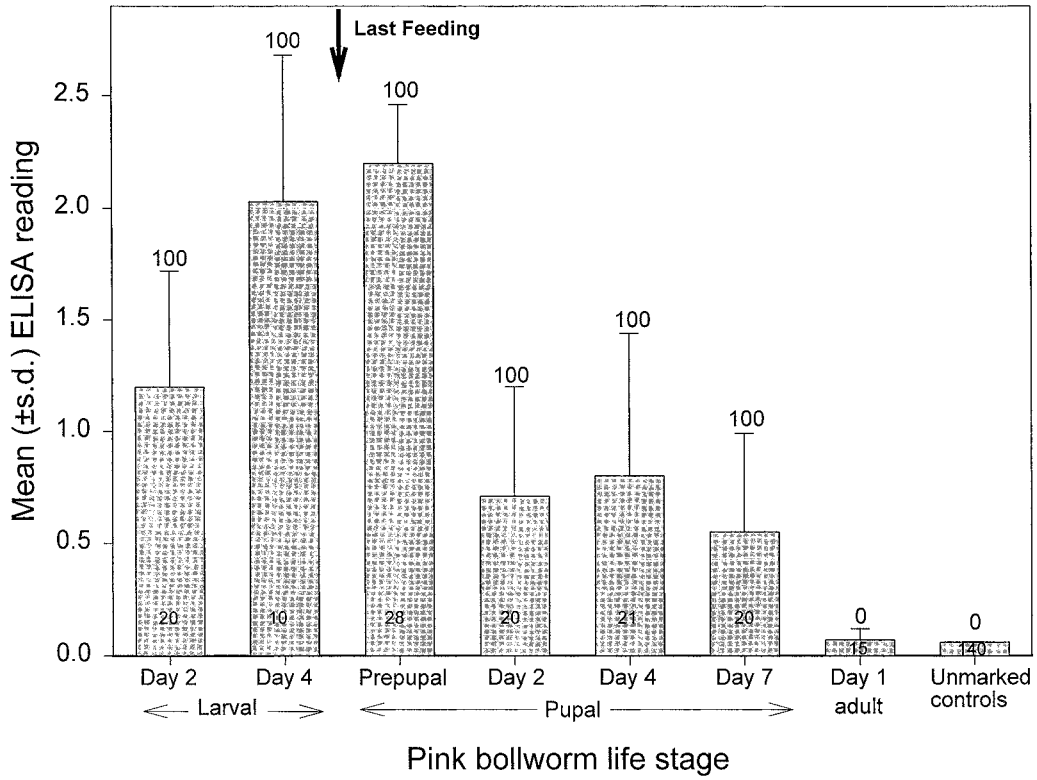

Figure 1. Mean ± s.d. ELISA readings for the retention of rabbit IgG in the various pink bollworm life stages in which fourth instar larvae had fed on an artificial diet containing rabbit IgG. The numbers above the error bars are the percentage of individuals scoring positive for the presence of rabbit IgG. The numbers inside the gray bars are the sample sizes for each treatment. The arrow indicates the point that pink bollworm are no longer in contact with the protein-enriched diet. The unmarked negative controls for each treatment were pooled.

adults. Approximately three days prior to adult emergence, pink bollworm pupae (≈200) were submerged into 5.0 ml of a 5.0 mg/ml reagent grade rabbit IgG (I-5006, Sigma Chemical Co., St. Louis, MO) solution for 1 min. The pupae were dried on filter paper for 3 h at 27 °C. Marked and unmarked pupae were collected and frozen one and three days after marking. The remaining pupae were individually placed in 30 ml disposable plastic containers (Shot Glass and Portion Cup, Jet Plastica, Hatfield, Penn.) and held in the environmental chamber described above. Moths that had emerged from marked and from unmarked control pupae were collected daily for ten days after emergence and analyzed by ELISA. The mean (± s.d.) ELISA readings and the percentage of pink bollworm scoring positive for rabbit IgG were recorded for each group.

Experiment 3: retention of protein on externally marked moths

Experiments were conducted to determine the retention of rabbit IgG on externally marked adults under field conditions and after exposure to sticky traps. One-day-old moths were placed in a 2.5-liter Tupperware® container and held for 15 min at 4 °C. The lid of each container had a 6.0 cm diameter hole covered with organdy fabric to facilitate air exchange. Moths were externally marked with technical grade rabbit IgG using a medical nebulizer. A nebulizer, a common medical device used to deliver inhaled medications, produces a very fine, fog-like mist (Hagler 1997b). Briefly, 1.0 ml of a water solution containing 25.0 mg of rabbit IgG was placed into the nebulizer. The hose of the nebulizer was attached to a standard laboratory air outlet and the mouth of the nebulizer was inserted into a 2.5 cm diameter hole (just slightly larger than the mouth of the nebulizer) in the side of the Tupperware container. The air outlet was turned on and the moths were 'fogged' with the rabbit IgG

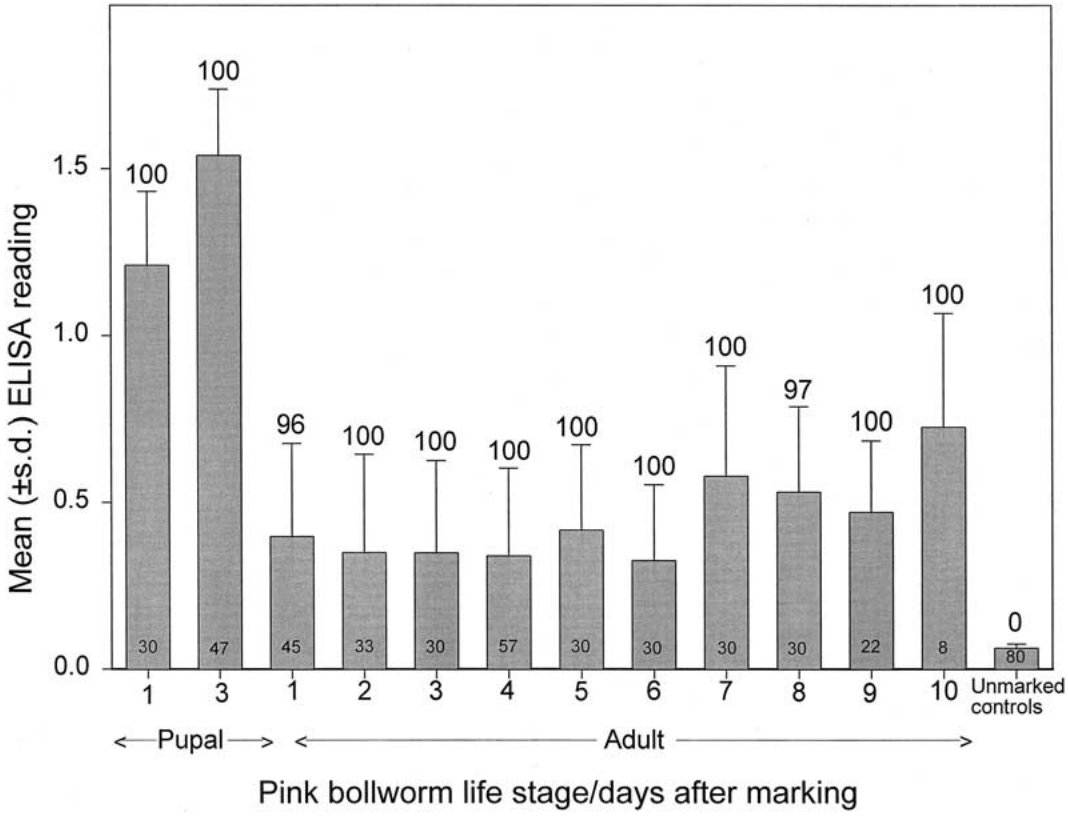

Figure 2. Mean ± s.d. ELISA readings for the retention of rabbit IgG on pink bollworm pupae submerged in a rabbit IgG solution and adults that emerged from marked pupae. The numbers above the error bars are the percentage of individuals scoring positive for rabbit IgG. The numbers inside the gray bars are the sample sizes for each treatment. The unmarked negative controls for each treatment were pooled.

solution for ≈2 min. The nebulizer was removed from the container and the 2.5 cm diameter hole in the container was plugged with a cork. The moths were kept in the marking container for 2 h and then subjected to the tests described below.

Field-cage retention test. A field test was conducted to determine how long rabbit IgG remains on externally marked moths. Adult males were marked by the fogging method described above. Just prior to release, a small group of moths (n = 31) were collected for ELISA analysis and the remaining moths were released at dusk in uninfested cotton plots enclosed in field cages (3.6 × 7.6 × 1.8 m) located at the Western Cotton Research Laboratory, Phoenix, Arizona. Two Delta traps (Scentry Biologicals, Billings, MT) (Foster et al., 1977) baited with pink bollworm pheromone (Biolure, Consep Membrane, Bend, OR) were placed in the cages overnight 3, 6, 9, and 12 days after the moths were released. The traps were removed from the cages the following morning (e.g., 4, 7, 10, and 13 days after marking) and each captured individual was carefully removed from the sticky trap for analysis. The mean (± s.d.) ELISA readings and the percentage of pink bollworm scoring positive for rabbit IgG were recorded for each group.

Sticky card test. A test was conducted to determine if rabbit IgG remained detectable on externally marked moths after capture on a sticky trap. Adult male and female pink bollworm (≈500) were marked by the fogging method described above. After 2 h, the moths were transferred to a 5.7-liter holding cage with cardboard sides, a plastic bottom, and a screened lid. Forty male and 40 female moths were removed from the cage after one, four, and seven days. The females were 'sprinkled' onto the sticky surface of a Delta trap and the males were placed into individual rearing cups. Both groups of moths were then held in the environmental chamber described above for 24 h. The trapped females and untrapped males were then analyzed by ELISA. The mean (± s.d.) ELISA readings

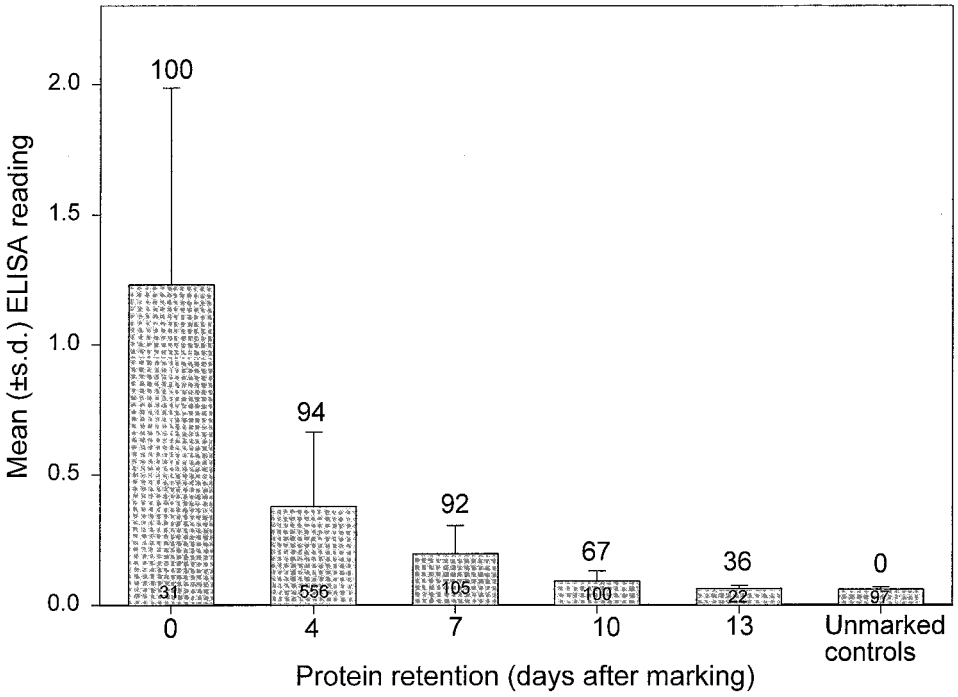

Figure 3. Mean ± s.d. ELISA readings for the retention of rabbit IgG on pink bollworm adults sprayed with rabbit IgG. The numbers above the error bars are the percentage of individuals scoring positive for rabbit IgG. The numbers inside the gray bars are the sample sizes for each treatment. The unmarked negative controls for each treatment were pooled.

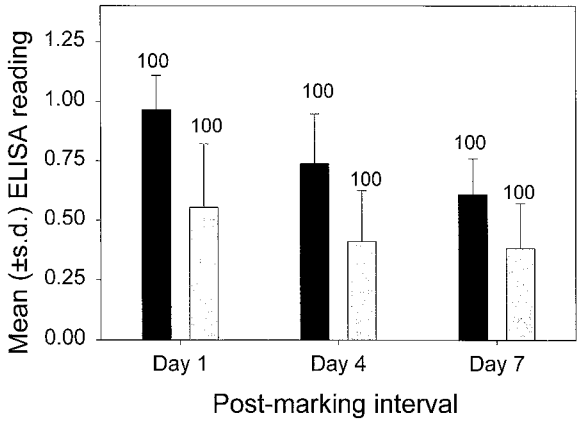

Figure 4. Mean ± s.d. ELISA readings for the retention of rabbit IgG on pink bollworm adults sprayed with rabbit IgG and then isolated in individual rearing containers (black bars) or captured on a sticky trap (gray bars). The numbers above the error bars are the percentage of individuals scoring positive for rabbit IgG (n = 40 per treatment). The pooled mean unmarked negative control value was 0.06 ± 0.01.

and the percentage of pink bollworm scoring positive for rabbit IgG were recorded for each group.

Experiment 4: contamination tests

Two tests were conducted to determine if externally marked moths transfer rabbit IgG to unmarked moths. The first test was conducted to determine if a communal group of marked moths transfer rabbit IgG to unmarked moths. The second test was conducted to determine if an individual marked male moth transfers rabbit IgG to an unmarked female.

Communal test. One-day-old adult males (≈450) were marked using the fogging method described above. After 2 h, the moths were transferred to the 5.7-liter cage described above. Additionally, 160 unmarked males possessing a mutant *white-eye* color and 160 unmarked females possessing a mutant *orange-eye* color were added to the cage. The caged moths were held under subdued light at 27 °C and 80% r.h. After 24 h, individuals from each treatment were removed from the cage every other day for ten days and then analyzed by ELISA. The mean (±s.d.) ELISA readings and the percentage of pink bollworm scoring positive for rabbit IgG were recorded for each group.

Paired test. One-day-old male pink bollworm moths were marked by the fogging method described above and then placed into a clean cage for 2 h. After 2 h, a single marked male was transferred to a 30 ml rearing cup containing an unmarked virgin female. The two moths in each container were placed into the environmental chamber described above for 48 h and then analyzed by the ELISA described above with one modification, the substrate incubation time was reduced to 30 min. The mean (±s.d.) ELISA readings and the percentage of pink bollworm scoring positive for rabbit IgG were recorded for each group.

Results

Experiment 1: transfer of the rabbit IgG mark between life stages

Pink bollworm larvae feeding on rabbit IgG-enriched diet and non-feeding prepupa yielded the highest ELISA responses (Figure 1). Furthermore, the protein marker was transferred to the pupal stage. Every pupa examined, regardless of its age, contained detectable amounts of rabbit IgG. However, rabbit IgG was not detected in any of the adults.

Experiment 2: retention of protein from marked pupae to adults

Externally marked pupa clearly contained more rabbit IgG than the adults that emerged from them (Figure 2). However, rabbit IgG was detected on 99% of the adults examined throughout the ten days of the experiment.

Experiment 3: retention of protein on externally marked moths

Field cage retention study. Almost all of the externally marked field released and recaptured adults contained detectable amounts of rabbit IgG after seven days (Figure 3). However, the retention of rabbit IgG on the moths declined after seven days. The average ELISA readings for those individuals collected ten and 13 days after release were similar to the readings yielded by the unmarked moths. Consequently, only about half of these individuals contained detectable amounts of rabbit IgG.

Sticky card study. The moths trapped for 24 h on sticky adhesive consistently yielded lower ELISA readings than the moths that were not trapped (Figure 4). However, all trapped moths contained detectable amounts of rabbit IgG.

Experiment 4: contamination tests

Communal test. The moths marked with rabbit IgG consistently yielded ELISA readings ten times higher than unmarked males and females (Figure 5). However, the majority of both sexes of unmarked moths contained detectable amounts of rabbit IgG after only three days of exposure to the marked moths.

Paired test. Two days after marking, all marked males contained detectable levels of rabbit IgG while only 4.4% of the unmarked females contained detectable levels of rabbit IgG (Figure 6).

Discussion

The insect marking procedure described here provides researchers with an alternative to conventional marking methods (Hagler & Jackson, 2001). Although we selected pink bollworm for this study, the procedures are applicable to most insect species. Protein marking and its accompanying ELISA are simple, inexpensive, and environmentally safe. Furthermore, the ELISA is sensitive and the anti-rabbit IgG is highly specific. Every unmarked pink bollworm examined in this study, regardless of its life stage, yielded a low ELISA reading. As a result, none of the unmarked pink bollworm yielded a false positive ELISA response. The low ELISA readings indicate that anti-rabbit IgG does not cross-react with any of the proteins present in pink bollworm. These results are similar to other cross reactivity tests of anti-rabbit IgG against other insect species (Hagler et al., 1992; Hagler, 1997a, b; Hagler & Jackson, 1998; DeGrandi-Hoffman & Hagler, 2000).

Pink bollworm larvae fed rabbit IgG-enriched diet did not retain the IgG protein in the adult stage. These results suggest that marking pink bollworm adults by incorporating rabbit IgG into larval diet is not a viable alternative to the conventional marking procedure of adding Calco Oil Red dye to larval diet (Graham & Mangum, 1971). The reason the protein did not transfer to adults is unknown and deserves further investigation.

The external mass marking technique of submerging pink bollworm pupae into rabbit IgG was an

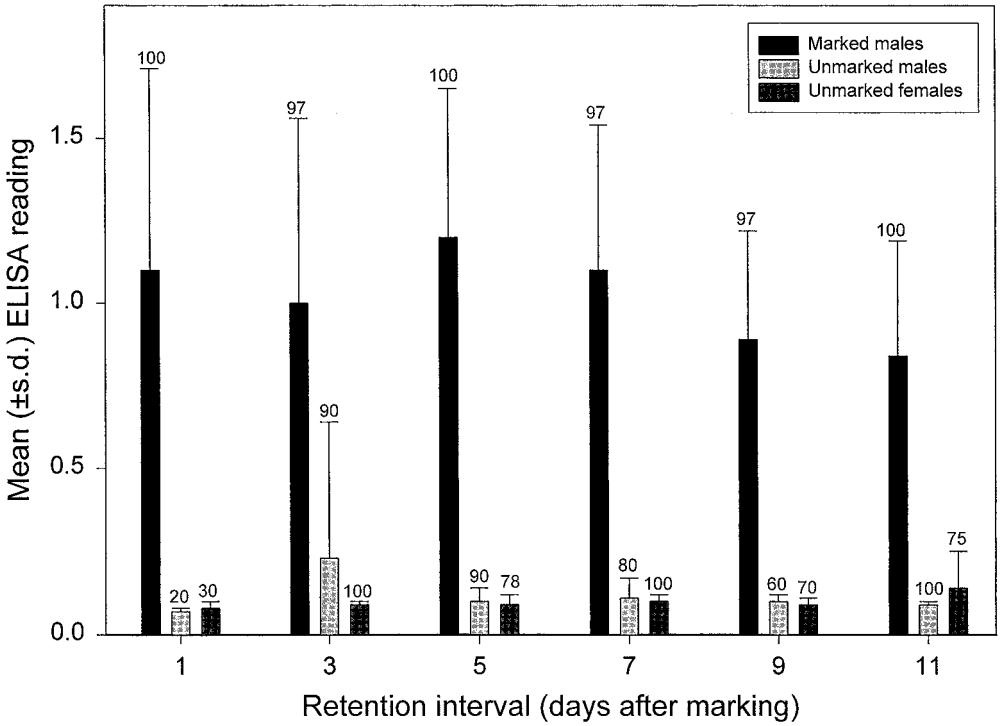

Figure 5. Mean ± s.d. ELISA readings for the retention of rabbit IgG on pink bollworm moths sprayed with rabbit IgG (black bars), unmarked males (gray bars), and unmarked females (white bars). The numbers above the error bars are the percentage of individuals scoring positive for rabbit IgG (n = 40, 10, and 10 for marked males, unmarked males, and unmarked females, respectively). The pooled mean unmarked negative control value was 0.06 ± 0.01.

excellent method for labeling both pupae and adults. Every individual examined, regardless of the life stage or age of the adult, yielded a positive ELISA response. Submersion could be a useful alternative to marking procedures that involve feeding larvae a labeled diet (Hagler & Jackson, 2001). Submerging pupae in rabbit IgG has several advantages over dye marking by larval feeding or directly spraying adult moths. First, non-mobile pupae are easy to handle and manipulate. Second, thousands of pupae can be marked in a few seconds using minimal amounts of rabbit IgG. Third, pupal marking in some situations might be less time consuming and less labor intensive than mixing a marker into an insect's diet. Finally, pupal marking is more practical than dye marking by larval feeding when artificial diets are not available for the insect species targeted for marking.

The external marking technique of spraying adults with a nebulizer was effective for labeling the majority (>92%) of adults for six days. Applying a greater concentration of rabbit IgG to the moths or using a different application method would probably yield a longer retention interval. We used a nebulizer in this experiment because it was effective for marking small parasitoids (Hagler, 1997b). However, a different application method might have yielded better results. For example, rabbit IgG and chicken IgG were retained well on *Hippodamia convergens* for over three weeks in the field after they were topically marked with a hand spray bottle (Hagler, 1997a).

A factor to consider for any mark-release-recapture study is the method used to recapture marked insects after they are released. It is important that the trapping method does not adversely affect the efficacy of the marker. The common method for capturing pink bollworm and many other insect species relies on various kinds of traps (e.g., yellow cards, pheromone traps, etc.) coated with sticky adhesive (e.g., Tanglefoot®) (Muirhead-Thomson, 1991). An examination of the retention of rabbit IgG on externally marked adults after exposure to a sticky trap showed that the ELISA readings were consistently lower for those moths trapped on the sticky adhesive. However, every trapped individual still contained detectable amounts of rabbit IgG.

Another factor to consider with any insect marking technique is whether the mark has the potential to pass laterally from marked to unmarked individuals. In some research situations it is desirable to have a marking technique that passes between individuals (Gu et al., 2001). For example, honey bees, *Apis mellifera* L. readily transfer rabbit IgG to unmarked nestmates (e.g., nurse bees, larvae, etc.) via trophallaxis. This attribute facilitated the examination of the flow of incoming nectar through a honey bee colony by feeding foraging honey bees a sugar solution containing rabbit IgG (DeGrandi-Hoffman & Hagler, 2000). Additionally, the trace element rubidium is sometimes used for mating studies because some insect species can pass rubidium to unmarked mates (Graham & Wolfenbarger, 1977; Van Steenwyk et al., 1978; Hayes, 1989). However, for most mark-release-recapture studies it is not desirable to have a mark that can transfer between individuals because this would lead to erroneous estimations of dispersal and population density. The two tests we conducted examined the possibility of rabbit IgG marked insects contaminating unmarked insects. For the first test, a relatively small group of unmarked moths were placed in a cage containing a large group of marked moths. The majority of both unmarked males and females were rapidly contaminated with rabbit IgG. The high proportion of contaminated moths was probably an artifact of the experimental design. For example, an enormous number of body and wing scales accumulated in the cage which contained many more moths per unit area than would be found in nature. For the second test, an unmarked female moth was placed in a small container with a marked male. Only 4.4% of the females were contaminated with rabbit IgG. These results indicate that there is a good chance for contamination of unmarked individuals in a crowded environment, but only a slight chance for contamination in an uncrowded environment.

Purified proteins, when combined with protein-specific antibodies have enormous potential for marking a wide variety of animals for ecological studies. Furthermore, proteins can be useful for backing up conventional marking procedures. For example, several insect mass rearing programs including pink bollworm, fruit fly, boll weevil, and screwworm rely on visible dusts or dyes for marking insects (Gast & Landin, 1966; Graham & Mangum, 1971; Coppedge et al., 1979; Schroeder & Mitchell, 1981). However, it is widely acknowledged that these visible markers are not always effective (EM pers. obs.). If a dye- or dust-marked insect was also marked with protein prior to its release, then a potentially false negative visible mark could be further examined in the laboratory by ELISA for the presence of a protein mark. A second mark on released individuals might provide a failsafe method for ensuring that 100% of the released insects are marked. In some cases, such as in sterile insect release programs, differentiation among marked and unmarked insects is so critical that a single false negative insect in a trap could trigger an unnecessary increase in the number of sterile moths released in the area of the trap or possibly trigger the use of pheromone or insecticide treatments. Such a double marking procedure might ultimately eliminate the need for costly control procedures.

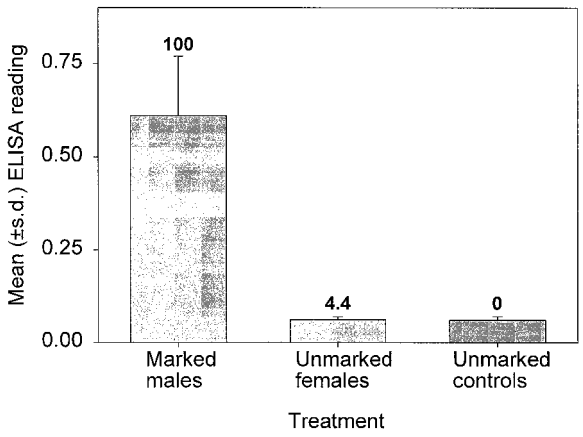

Figure 6. Mean ± s.d. ELISA readings for the retention of rabbit IgG on pink bollworm males sprayed with rabbit IgG and unmarked females after 48 h of exposure. The numbers above the error bars are the percentage of individuals scoring positive for rabbit IgG (n = 91 per treatment). The unmarked negative controls for each treatment were pooled.

Another potential use for protein marking is using multiple proteins to mark different groups of released insects (Hagler, 1997a). For example, we determined that rabbit IgG and chicken IgG antibodies do not cross-react. Therefore different IgGs can be used to mark different cohorts of individuals for studies of intercrop dispersal (Hagler & Naranjo, 1997).

While protein marking has many advantages over conventional insect markers, we would be remiss if its limitations were not discussed. Protein-marked insects must be chemically analyzed by ELISA for detection. Protein detection by ELISA is obviously more difficult, costly and time-consuming than simply detecting a dye or a dust on an insect by visual inspection. Furthermore, the insect specimen must be crushed prior

to the ELISA. This feature eliminates the possibility of using the recaptured insect for any further analyses.

In summary, we plan to continue using vertebrate-specific proteins for tracking the movement of insect pests and their natural enemies. Furthermore, studies are underway to determine if protein marking affects the development and behavior of pink bollworm larvae and adults. Marking pink bollworm moths by submerging the pupae into rabbit IgG has enormous potential as a mass marking technique for lepidopterous pests. Additionally, externally marking adults with a topical solution of rabbit IgG is also a viable marking procedure. The best method for applying protein to mark insects ultimately depends on the nature of the experiment, the insect under investigation, the insect life stage needing marked, and the preference of the investigator.

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